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Zeitz, J O ; Bucher, S ; Zhou, X ; Meile, L ; Kreuzer, M ; Soliva, C R

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## Inhibitory effects of saturated fatty acids on methane production by methanogenic *Archaea*

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**ABSTRACT.** The present study investigated the inhibitory effects of saturated fatty acids on methanogenesis in *Archaea*, and whether or not competitive inactivation of the methanogens' coenzyme M (HS-CoM) is involved in the inhibition. Strains tested in batch cultures were *Methanosarcina barkeri*, *Methanosarcina mazei*, *Methanococcus voltae*, all incubated at 37°C, and *Methanothermobacter thermoautotrophicus*, incubated at 65°C. The fatty acids C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub> and C<sub>18</sub> were supplemented at 1 mg · ml<sup>-1</sup> in cultivation medium. The methanogens were susceptible to C<sub>10</sub> and C<sub>12</sub>, and less so to C<sub>14</sub>. Only *M. thermoautotrophicus* was affected by C<sub>18</sub>. In *M. mazei* cultures, excessive HS-CoM did not prevent the action of C<sub>14</sub> which might suggest that competitive inhibition of HS-CoM is not the reason for the SFA-induced effect on methanogenesis. The results indicate that, as a prerequisite to inhibit methanogenesis in *Archaea*, medium- and long-chain saturated fatty acids have to be at least partially molten.

### Introduction

The *Archaea* colonizing the rumen have attracted attention as they form the greenhouse gas methane (CH<sub>4</sub>) thus contributing to global warming (Hook et al., 2010). Dietary fats rich in saturated medium-chain length fatty acids (MCFA) have been shown to suppress CH<sub>4</sub> formation *in vivo* (Machmüller and Kreuzer, 1999). At 39°C, lauric (C<sub>12</sub>) and myristic acid (C<sub>14</sub>), two MCFA, are anti-methanogenic both in ruminal fluid (Dohme et al., 2001) and in sludge (Koster and Cramer, 1987), while saturated fatty acids (SFA) of longer-chain length such as palmitic acid (C<sub>16</sub>) and stearic acid (C<sub>18</sub>) did not exert this effect *in vitro* (Dohme et al., 2001; Soliva et al.,

2004; Zhang et al., 2008) and *in vivo* (Hristov et al., 2009). However, findings are not always consistent, e.g. C<sub>12</sub> and C<sub>14</sub> do not necessarily inhibit C<sub>14</sub> emissions *in vivo* (Hristov et al., 2009, 2011). Effective inhibition of ruminal methanogenesis by SFA requires knowledge about factors influencing the efficiency of CH<sub>4</sub> mitigation and the underlying modes of action. In the present study, as a first hypothesis it was tested whether the anti-methanogenic effect of SFA in methanogenic *Archaea* is affected either by SFA chain length, which is related to SFA melting temperature, or by cultivation temperature or both; a thermophilic methanogen species was employed for investigations at high cultivation temperature. To effectively target all methanogens, CH<sub>4</sub> suppres-

sors have to affect cell structures or metabolic functions common to all methanogens, although methanogens differ in composition and structure of their cell envelopes (Albers and Meyer, 2011) as well as utilize different substrates, metabolic pathways and enzymes (Thauer et al., 2008). The second hypothesis tested was, therefore, that SFA act anti-methanogenic in methanogenic *Archaea* independent of the metabolic pathway used to form  $\text{CH}_4$ . For that purpose, *Methanosarcina mazei*, able to grow without  $\text{H}_2$  and  $\text{CO}_2$  as substrates (Hovey et al., 2005), was cultivated on methanol to test whether this methanogenic pathway is also inhibited. A key coenzyme for any kind of methanogenesis and thus common to all methanogenic *Archaea* is the methyl-coenzyme M reductase (MCR) (Thauer, 1998). The genes encoding MCR are expressed to the same level irrespective if *M. mazei* forms  $\text{CH}_4$  from acetate or from methanol even though numerous, mainly methanol-pathways specific, genes show different expression levels in the cells grown on methanol as compared to the cells grown on acetate (Hovey et al., 2005). This enzyme is involved in the release of  $\text{CH}_4$  from methyl-coenzyme M by formation of a heterodisulfide of 2-mercaptoethanesulfonic acid (HS-CoM), i.e. coenzyme M, and coenzyme B (Thauer, 1998). The SFA have a certain structural similarity to coenzymes M and B thus maybe acting as competitors for the position in the hydrophobic channel of the MCR. If this were true, an excess of HS-CoM would actively counteract this competition. The third hypothesis tested was therefore that the anti-methanogenic action of SFA is mediated through competition with coenzyme M, thus inactivating MCR (Thauer, 1998) and, in consequence,  $\text{CH}_4$  formation.

## Material and methods

### Experimental microbes and cultivation

All methanogenic strains investigated were obtained as live cultures from DSMZ (www.dsmz.de) delivered in cultivation media. *Methanosarcina barkeri* DSM 800 and *M. mazei* DSM 7222 were both isolated from an anaerobic sewage digester, and *Methanococcus voltae* DSM 1537 was isolated from mud. All had a temperature optimum between 35 to 37°C and were cultivated at 37°C. The thermophilic strain *Methanothermobacter thermoautotrophicus* DSM 1053, isolated from sewage sludge with a temperature optimum of 55 to 70°C was cultivated at 65°C. The intention for selecting these strains was to include non-thermophilic strains representing four different methanogenic orders to get an overview if the anti-methanogenic effect of SFA might

occur in all methanogens. Cultures of *M. barkeri* and *M. mazei* were shaken with a Kühneshaker Labtherm (Adolf Kühner AG, Birsfelden, Switzerland) at 80 rpm during cultivation. Anaerobic cultivation was performed in 120-ml cultivation flasks containing 30 ml of the respective, strain-specific cultivation media as described by DSMZ. Different from the DSMZ protocols, Na-EDTA ( $14.2 \mu\text{mol} \cdot \text{l}^{-1}$ ) instead of nitrilotriacetate was used for the cultivation medium of *M. voltae*. Ruminant fluid from a rumen-cannulated cow was clarified by filtering through four layers of medicinal gauze and subsequent centrifugation for 15 min at 4000 g (Varifuge® K, Heraeus, Osterode, Germany). Then the supernatant was filtered five times using filters with pore sizes of 20, 2.5, <2.0, <0.7, and 0.2  $\mu\text{m}$ . Heat stable solutions of other media ingredients were sterilized in a batch autoclave (Sauter, Belimed Sauter AG, Sulgen, Switzerland) for 20 min at 121°C. Heat susceptible solutions were filtered through a 0.2  $\mu\text{m}$  Minisart-plus filter (Sartorius AG, Göttingen, Germany) and a vacuum-sterile filter (VWR 250 ml system capacity, 0.2  $\mu\text{m}$  pore size, VWR International AG, Dietikon, Switzerland). Media reagents were prepared in an anaerobic chamber using boiled oxygen-deprived distilled water (Coy Laboratory Products, Grasslake, USA).

Culture development was monitored by analyzing  $\text{CH}_4$  formation. This variable had been found to vary in parallel to changes in optical density, i.e. growth, in own preceding investigations. Before the start of the experiment, 20 to 50  $\mu\text{l} \cdot \text{ml}^{-1}$  of previously established cultures – from now on named ‘pre-cultures’ – were used to inoculate fresh medium when  $\text{CH}_4$  formation was in the mid-exponential phase.

The SFA tested were  $\text{C}_{10}$ ,  $\text{C}_{12}$ ,  $\text{C}_{14}$  and  $\text{C}_{18}$ , each of a purity of at least 97% (Fluka Chemie AG, Buchs, Switzerland). Concentrated SFA stock solutions ( $30 \text{ mg} \cdot \text{ml}^{-1}$ ) were prepared without solvent with cultivation medium alone, autoclaved, and stored at 65°C before being added to the methanogen cultures. The dosage selected was  $1 \text{ mg} \cdot \text{ml}^{-1}$  cultivation medium as this was equivalent to the level that proved effective in a previous *in vitro* experiment with bovine ruminal fluid (Soliva et al., 2003). The sodium 2-mercaptoethanesulfonate (HS-CoM) was obtained from Sigma-Aldrich (BioXtra, Buchs, Switzerland) with  $\geq 98.0\%$  purity.

### Experiment 1

The experimental cultures were initiated with concentrations of 28, 17 and 50  $\mu\text{l} \cdot \text{ml}^{-1}$  of pre-cultures of *M. barkeri*, *M. voltae* and *M. thermoautotrophicus*, respectively, with pre-culture optical densities, measured at 600 nm, of 0.77, 0.86 and 0.11, respectively, under a 80%

H<sub>2</sub> : 20% CO<sub>2</sub> atmosphere (Pangas AG, Dagmar-sellen, Switzerland) and an initial headspace gas pressure of 200 to 250 kPa. The gas mixture in the headspace was renewed every 24 h. The SFA were supplemented to the respective cultures when methanogens had reached the mid-exponential phase. *M. barkeri* was exposed to C<sub>12</sub> and C<sub>14</sub>, *M. voltae* to C<sub>10</sub>, C<sub>14</sub> and C<sub>18</sub>, and *M. thermoautotrophicus* to C<sub>12</sub>, C<sub>14</sub> and C<sub>18</sub>. This approach accounted for the limitation in numbers of incubation bottles to be handled while still having always one reference fatty acid (C<sub>14</sub>). After adding SFA, CH<sub>4</sub> formation was measured for at least 24 h. Cultivations were performed in triplicate. Control cultures consisted of the respective methanogenic strains without exposure to SFA. Subsequently, it was tested whether the SFA-effects exhibited on the strains were reversible. For that purpose, 28, 17 and 50 µl · ml<sup>-1</sup> of the experimental and control cultures, were taken 24 h after the exposure to SFA and cultivated in fresh medium without adding SFA.

## Experiment 2

In the second experiment, *M. mazei* was grown in triplicate in N<sub>2</sub> atmosphere under a headspace gas pressure of 100 kPa on methanol (0.25 mol · l<sup>-1</sup> medium) as sole substrate for methanogenesis. 17 µl · ml<sup>-1</sup> of pre-culture in the mid-exponential phase was used for inoculation. For comparative reasons, C<sub>14</sub> was chosen as this was the only SFA used with each of the strains investigated in Experiment 1. The cultures were exposed to 1 mg C<sub>14</sub> · ml<sup>-1</sup> medium. Additionally, either 0 or 1.4 mg of HS-CoM · ml<sup>-1</sup> was supplemented to the incubation flasks. This is equivalent to a ratio of 1 mol C<sub>14</sub> : 2 mol HS-CoM, which was higher than what is required according to stoichiometric calculations by the methanogens to form the amount of CH<sub>4</sub> measured in pre-cultures and therefore, assumed to be available in excessive amounts.

## Laboratory and statistical analysis

The gas volume produced in the batch cultures was calculated from headspace volume, overpressure (measured by the manometer GDH 200-13, Greisinger Electronic GmbH, Regenstauf, Germany), and temperature applying the law of the ideal gas. A volume of 0.15 ml gas was collected from the headspace of the cultivation flasks and analyzed for its CH<sub>4</sub> concentration using a gas chromatograph (model 6890N, Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and a 234 mm × 23 mm column (80/100; 166 mesh; Porapak Q, Fluka Chemie AG, Buchs,

Switzerland). Methane formation rate was calculated from gas volume produced and changes occurring in CH<sub>4</sub> concentration in the meantime between two measurement points.

Analysis of variance was performed using the MIXED procedure of SAS (version 9.1 of 2003; SAS Institute Inc., Cary, NC) with the repeated statement to compare control and SFA-supplemented cultures at each measurement point. Multiple comparisons were done using Tukey's procedure. In order to evaluate the reversibility of SFA-inhibited cultures at each measuring point, the TTEST procedure of SAS was used. In order to evaluate whether the culture CH<sub>4</sub> formation was different from zero, the one-sample t-test was applied. For pairwise comparisons between those treatment groups where a certain culture recovery occurred the two-sample t-test was used. Differences were declared significant at P < 0.05.

## Results

### Experiment 1

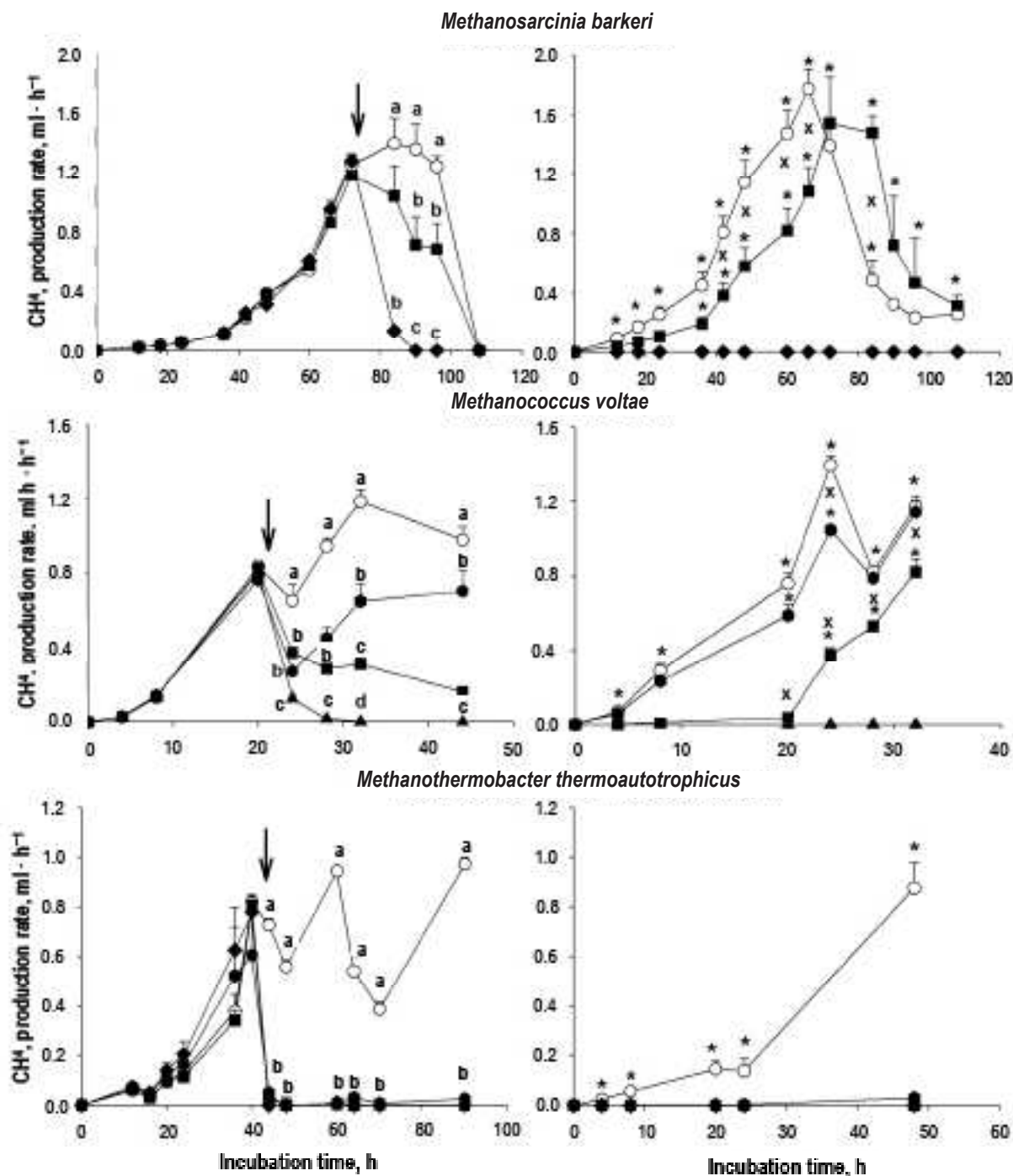
The CH<sub>4</sub> formation by *M. barkeri* was significantly decreased by 90% within 12 h and ceased after 18 h subsequent to adding C<sub>12</sub> (Figure 1, top). No CH<sub>4</sub> formation was detected after transferring C<sub>12</sub>-inhibited *M. barkeri* to new, C<sub>12</sub>-free medium. With C<sub>14</sub>, 18 h after addition, CH<sub>4</sub> formation rate was significantly decreased by 47% compared to that of the control. In the reversibility period, CH<sub>4</sub> formation of previously C<sub>14</sub>-treated cultures was retarded by about 6 to 12 h.

In cultures of *M. voltae*, the supplementation of C<sub>10</sub> suppressed CH<sub>4</sub> formation completely and irreversibly after just 8 h of cultivation (Figure 1, middle). C<sub>14</sub> decreased the CH<sub>4</sub> formation rate of *M. voltae* by 69% and 84% when measured 8 h and 24 h after the supplementation, respectively. CH<sub>4</sub> formation was delayed when being re-cultured.

The supplementation of C<sub>18</sub> had an immediate inhibitory effect on CH<sub>4</sub> formation rate (-58%) 4 h after C<sub>18</sub> addition compared to control, but increased again later and no adverse effect on the recovery of *M. voltae* was observed.

The supplementation of any SFA, that is C<sub>12</sub>, C<sub>14</sub>, and C<sub>18</sub>, almost completely terminated CH<sub>4</sub> formation of *M. thermoautotrophicus* within 4 h after supplementation (Figure 1, bottom). CH<sub>4</sub> formation ceased after 8 h of SFA exposure, except for C<sub>18</sub>, where CH<sub>4</sub> formation still amounted to 2 to 5% of that of the control treatment. None of the SFA-treated *M. thermoautotrophicus* cultures recovered in SFA-free medium.





**Figure 1.** Inhibition of methanogenic *Archaea* by different fatty acids (left) and their reversibility (right) in Experiment 1. Fatty acids used were  $C_{12}$  and  $C_{14}$  in *Methanosarcinia barkeri*,  $C_{10}$ ,  $C_{14}$  and  $C_{18}$  in *Methanococcus voltae*, and  $C_{12}$ ,  $C_{14}$  and  $C_{18}$  in *Methanothermobacter thermoautotrophicus*. —○— not supplemented control, —●— supplementation of  $C_{10}$ , —◆— supplementation of  $C_{12}$ , —■— supplementation of  $C_{14}$ , —▲— supplementation of  $C_{18}$ , ↓ — time point of fatty acids supplementation. Means with unequal letters (a, b, c, d) are different at  $P < 0.05$ . Asterisks denote means significantly different from 0 at  $P < 0.05$  (1-sample t test) and × denotes a significant difference ( $P < 0.05$ ) either between control and  $C_{14}$  or between control and  $C_{18}$  (2-sample t test). Bars represent standard errors

## Experiment 2

Following  $C_{14}$  addition, cultures of *M. mazei* were inhibited by 91% after 6 h, and completely after 24 h, compared to the control (Figure 2, left) both with and without an excessive amount of HS-CoM. None of the treated *M. mazei* cultures recovered in  $C_{14}$ -free medium. One of the three control cultures

showed lower  $CH_4$  formation in the recovery period compared to the other control cultures, resulting in high standard errors in this treatment (Figure 2, right), thus preventing the difference to both  $C_{14}$ -containing treatments to become significant.

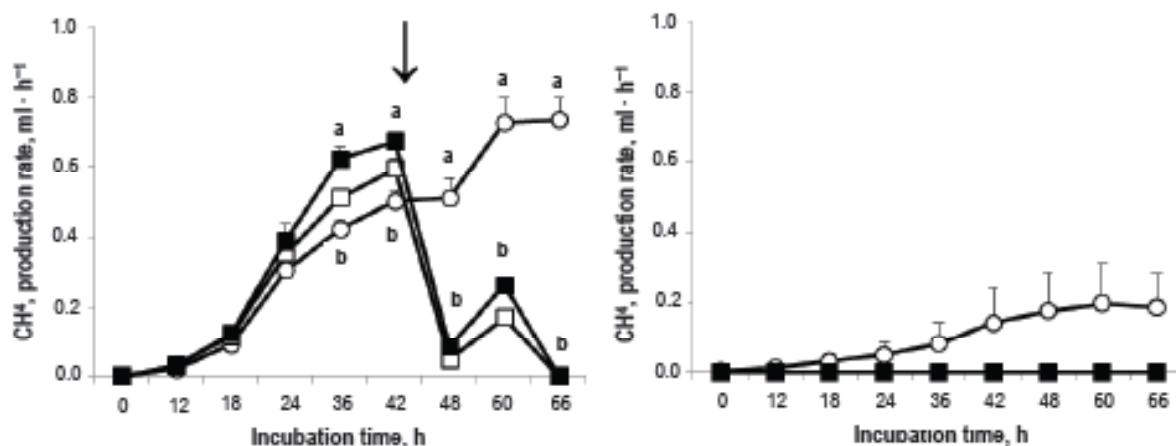


Figure 2. Inhibition of *Methanosarcina mazei* by C<sub>14</sub>, either with or without 2-mercaptoethanesulfonic acid (HS-CoM) supplementation (left) and its reversibility (right) in Experiment 2. ○ — not supplemented control, □ — supplementation of C<sub>14</sub>, ■ — supplementation of C<sub>14</sub> and HS-CoM ↓ — time point of supplementation of fatty acids. Means with unequal letters (a, b, c, d) are different at  $P < 0.05$ . Bars represent standard errors

## Discussion

### Importance of fatty acid chain length, cultivation temperature and substrate for the anti-methanogenic effect of SFA on the Archaea

At 37°C, the anti-methanogenic effect of SFA in methanogenic *Archaea* decreased with increasing chain length ( $C_{10} = C_{12} > C_{14} > C_{18}$ ) in the present study. A longer-chain length corresponds to increasing melting temperatures, namely 31, 45, 58, and 69°C for C<sub>10</sub>, C<sub>12</sub>, C<sub>14</sub>, and C<sub>18</sub>, respectively (Dohme et al., 2000). With increasing melting points the SFA are less well distributed in the cultivation medium at 37°C. Therefore, fewer cells might have been in contact with C<sub>18</sub> as compared to C<sub>14</sub> and especially to C<sub>12</sub>, which would allow the C<sub>18</sub>-treated cultures to recover faster. It was obvious that recovery was not observed when the SFA had stopped CH<sub>4</sub> formation completely thus indicating that cell death occurred. Methane formation during recovery has taken place at a slower velocity in cases where the previous anti-methanogenic effect had been more serious. A higher percentage of dead cells in the inocula from SFA-supplemented cultures might have contributed to this effect; however, cell viability was not tested in the present study. It could not be avoided that small amounts of SFA were carried over with the previously SFA-treated culture. However, then the concentration of SFA is 30-fold lower, which would not explain the massive delay in CH<sub>4</sub> formation found in the recovery period. Although the contact of SFA with the methanogenic cells might be a prerequisite, molten SFA do not necessarily have

an adverse effect on methanogenesis. For instance, caprylic acid (C<sub>8</sub>) supplemented to complete diets had no anti-methanogenic effect in ruminal fluid *in vitro* (Dohme et al., 2001; Abel et al., 2002) even though it is completely molten at 39°C. The reason for this is unclear. However, when the entire rumen microbial community and complete diets are involved, microbial and substrate interactions are much more complex and other factors of influence might be decisive and could prevent SFA-induced inhibition. It has also been shown that bacteria previously exposed to C<sub>18</sub> can get resistant to C<sub>18</sub> addition within hours (Laser, 1952), which might contribute to explain the recovery of *M. voltae* cultures exposed to C<sub>18</sub>.

At 65°C, *M. thermoautotrophicus* was clearly and irreversibly inhibited by C<sub>12</sub>, C<sub>14</sub>, and C<sub>18</sub>. The initiation of the C<sub>18</sub>-induced effect was slightly retarded compared to that of the other two fatty acids which might reflect that C<sub>18</sub> is not completely molten at 65°C. C<sub>18</sub> was also found to inhibit methanogenesis in cattle manure incubated at 55°C (Angelidaki and Ahring, 1992). However, composition and structure of the archaeal cell envelope differ within orders, which may include a response to habitat temperatures (Albers and Meyer, 2011) and result in a different susceptibility of *M. thermoautotrophicus* as compared to the strains grown at 37°C. It was not possible to successfully cultivate strains of two ruminal methanogens, *Methanomicrobium mobile* and *Methanobrevibacter ruminantium*, which were originally part of the study. Therefore, further studies have to demonstrate whether an apparent effect of temperature is really mediated by a different aggregate state of the SFA in ruminal methanogens.

## Potential modes of action regarding the inhibitory effect of the saturated fatty acids

The cell envelopes, especially the cell membranes, are probable target sites where effects of SFA are expected to occur in a range of microorganisms (Desbois and Smith, 2010). The fatty acids might also directly affect enzymes or other functional features of the methanogenic cell. This has already been proposed for enzymes and transport proteins in bacterial cell membranes (Desbois and Smith, 2010). In the present study, it was tested whether the MCR, a key enzyme in methanogenesis and common to all methanogenic *Archaea* (Thauer, 1998), is specifically inhibited by SFA, i.e. if an excess of HS-CoM, the coenzyme for the MCR and substrate analogon to SFA, would actively counteract this competition. However, in *M. mazei* an excessive amount of HS-CoM did not prevent the CH<sub>4</sub>-mitigating action of C<sub>14</sub> at all, suggesting that this is not the reason for the SFA-induced effect on methanogenesis. It still has to be tested if SFA are acting competitively with coenzyme B.

## Conclusions

Knowledge on the mode of action of SFA on methanogens is essential for developing effective and sustainable CH<sub>4</sub> mitigation tools, as they are intensively searched for (Hook et al., 2010). The present study demonstrated that CH<sub>4</sub> formation from different substrates and in different species of methanogenic *Archaea* is inhibited by SFA and is dependent on SFA chain length at 37°C. There are indications that the anti-methanogenic effect of SFA which are not (completely) molten at 37°C is increasing with increasing cultivation temperature, but future studies have to exclude that the increasing anti-methanogenic effect of SFA at 65°C in cultures of *M. thermoautotrophicus* is due to a specific susceptibility of this strain. Further studies with ruminal methanogens are necessary. The presence of a competitive inhibition of the methyl coenzyme M reductase by SFA can be excluded with high probability. The mechanism(s) causing CH<sub>4</sub> inhibition still awaits identification. This is also true for the reasons underlying the differences found between the methanogenic strains tested. The most promising sites to investigate, and to eventually understand the mode of action of the SFA-induced CH<sub>4</sub> mitigation, are the cell envelopes of the *Archaea*.

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